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Enhancing effect of a protein from *Lonomia obliqua* hemolymph on recombinant protein production

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Abstract Gene expression in animal cells allows large scale production of proteins used for either structure and function studies or therapeutic purposes. Maximizing recombinant protein production is necessary to optimize cell growth and protein expression. Some studies have demonstrated the presence of pharmacologically active substances in insect hemolymph. In this work, we have identified and purified a protein from *Lonomia obliqua* hemolymph able to increase the production of the rabies virus glycoprotein, expressed in *Drosophila melanogaster* S2 cells, by about 59%.

Keywords Insect cells · Recombinant · *Lonomia obliqua* · Purification · Rabies · Hemolymph

Abbreviations

FACS	Fluorescence-assisted cell sorting
FBS	Fetal bovine serum
rRVGP	Recombinant rabies virus glycoprotein

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Introduction

Gene expression in animal cells allows production of proteins used for either structure and function studies or therapeutic purposes. The optimization of cell growth and protein expression requires maximizing recombinant protein production, which has been carried out using several strategies, such as the addition of selective nutrients, stimulating agents and genetic and metabolic manipulation. However, these systems can be quite costly. Process costs can be reduced by either replacing certain components of the culture medium, which will result in high cell densities at lower costs and less operation complexity, or by using substances which promote cell growth, cell protection or even cell productivity. Some studies have demonstrated the presence of pharmacologically active substances in insect hemolymph (Shiotsuki et al. 2000; Yamamoto et al. 1999; Guerrero et al. 1999; Jiang et al. 1999; Rosenfeld and Vanderberg 1998; Hamdaoui et al. 1998; Maranga et al. 2003; Huberman et al. 1979; Lin et al. 1998; Kurata et al. 1994; Jones et al. 1993; Zhu et al. 2000; Lowenberger et al. 1999; Lamberty et al. 1999; Gross et al. 1998; Johns et al. 1998; Lanz-Mendoza et al. 1996; Peters et al. 1993; Souza et al. 2005). The supplementation of cultures with hemolymph proteins has had a positive effect on viral replication (Souza et al. 2005; Rhee and Park 2000; Kanaya and Kobayashi 2000) and recombinant protein production (Woo et al. 1997). Recently, Kanaya and Kobayashi

(2000), isolated and characterized a protein from silkworm hemolymph, able to increase the activity of a recombinant protein (luciferase) by approximately 6,000 times. Nevertheless, few studies have succeeded in isolating and characterizing the factors involved in these effects (Nussbaumer et al. 2000; Shishikura et al. 1996, 1997; Moon et al. 1995; Ochanda et al. 1992). These factors, once identified and isolated, can be of great importance in the optimization of cell growth, viral replication or recombinant protein production, leading to more efficient cell cultivations and to final products at lower costs. The objective of this work was to identify, isolate and characterize the protein(s) of the hemolymph from *Lonomia obliqua* that are likely to influence cell growth and recombinant rabies glycoprotein production in cultures of transfected S2 cells.

Materials and methods

Cell line and culture conditions

Drosophila melanogaster Schneider 2 (S2) cells expressing recombinant rabies virus glycoprotein (rRVGP) were grown in 100 ml Schott flasks containing TC-100 medium (Gibco), supplemented with 10% fetal bovine serum (FBS), or SF-900 serum free medium. The flasks were inoculated with S2 cells to provide an initial concentration of 0.5–1 $\times$ 10^6 cells/ml (TC-100 medium) or 5 $\times$ 10^6 cells/ml (SF-900 medium). The cultures were then incubated at 29°C and at 100 rpm in a incubator shaker. S2 cells expressing rRVGP was kindly provided by Yokomizo et al. (2007).

Hemolymph collection

The hemolymph of *Lonomia obliqua* was collected from sixth instar larvae after setae had been cut off. The collected hemolymph was clarified by centrifugation at 1,000 g for 10 min. Afterwards, the supernatant was heat-treated at 60°C for 30 min. The heat-treated hemolymph was then filtered through a 0.2 μm membrane and stored at 4°C.

Hemolymph fractionation by chromatography

After centrifugation and filtration, 6 ml of hemolymph were further fractionated by gel filtration chromatography using an AKTA Purifier chromatography system equipped with a Hi-prep 26/60 Sephacryl 200 column (Amersham Pharmacia Biotech) at a flow rate of 1 ml/min. The elution was monitored at 280 nm and 120 fractions (4 ml each) were collected. The fractions were then analyzed by SDS-PAGE and added to transfected S2 cell cultures for studies on cell growth and recombinant protein production.

Enhancing effect of hemolymph on recombinant protein production

In order to investigate the enhancing effect of hemolymph on recombinant protein production, whole hemolymph and hemolymph protein fractions were added to the culture medium to a final concentration of 1% (v/v) at the time of inoculation. Culture samples were taken daily and examined by FACS and confocal microscopy to determine the percentage of rRVGP-expressing cells and by ELISA to quantify the amount of protein produced.

Analytical procedures

Flow cytometry

Recombinant protein expression was quantitatively evaluated by flow cytometry. On a daily basis, samples of cultures containing transfected or non-transfected S2 cells, supplemented or non-supplemented with hemolymph (whole or fractions) were collected, washed twice with PBS (pH 7.4) and centrifuged at 800 g for 5 min. The pellet was stained with IgG anti-GPV antibody (Institute Pasteur). After 10 min, cells were centrifuged (1,000 g/5 min) and the pellet was resuspended in 1 ml of FACS buffer. Samples were processed on a Becton Dickinson FACSort equipped with an Ar laser (excitation and emission wavelengths were 488 and 620 nm, respectively). Ten thousand events were analyzed per sample.

rRVGP expression analysis by ELISA

The recombinant GPV levels produced by S2Ac-GPV2 cells were estimated by ELISA (Institute Pasteur, Paris) as described by Perrin et al. (1996). Transfected S2 cells expressing recombinant GPV

were centrifuged at 1,000 rpm for 5 min. The cell pellet was recovered and the cells were lysed with a lysing buffer. The lysate was then centrifuged at 10,000 rpm for 10 min and the supernatant was used to detect and quantify the protein. The immunofluorescence reaction was performed with FITC-labeled D1 mAB anti-RGPV (1:400) for 1 h at 37°C.

Determination of rRVGP-expressing S2 cells by Confocal microscopy

S2 cells, stained with anti-GPV antibody, were analyzed using a Bio-rad MRC-600 microscope equipped with a Kr/Ar Laser (1% laser transmission, 60% confocal aperture and 10× and 40× objectives). Excitation filters for 488 and 543 nm as well as emission filters for 505 nm were used.

Measurement of cell viability

Culture samples were taken daily and cell concentration was measured using a hemocytometer. Cell viability was determined by the trypan blue exclusion test under light microscopy.

Results

Hemolymph effect on cell growth

One of the main objectives of this work was to observe the effect of hemolymph supplementation on S2 cell growth. S2 cells were initially adapted to growth in SF-900 serum free medium or in TC-100 culture medium supplemented with bovine fetal serum. While SF-900, a high-cost complex medium, was chosen for being able to maintain high cellular densities (2×10^7 cells/ml), TC-100 basic medium was chosen for this study so that interference of medium components in rRVGP production would be minimized.

Figure 1a shows the growth profiles of non-transfected S2 cells grown in whole hemolymph supplemented (1% v/v) and non-supplemented culture media. As it can

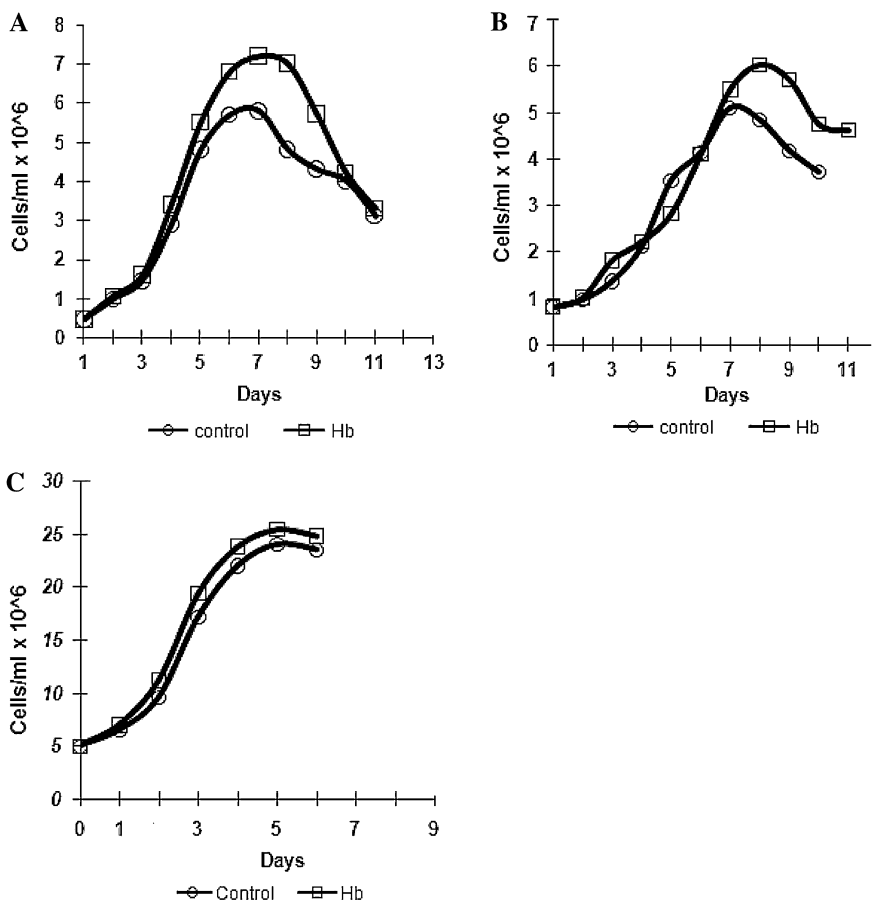

Fig. 1 Kinetics of S2 cell cultures performed in Schott flasks with TC-100 (**a**, **b**) and SF-900 (**c**) media. Flasks were inoculated with non-transfected (**a**) and transfected (**b**, **c**) S2 cells to provide an initial concentration of 0.5–1 $\times$ 10^6 cells/ml (**a**, **b**) or 5 $\times$ 10^6 cells/ml (**c**). Whole hemolymph (1% v/v) was added to the cultures at the time of inoculation. The cultures were conducted at 100 rpm and 29°C. Samples were taken daily and the viable cell number was determined using the trypan blue exclusion test

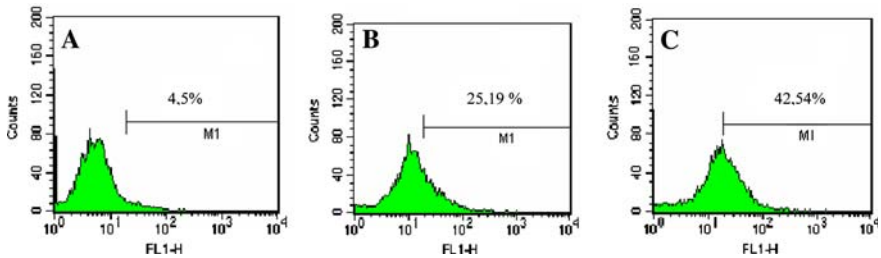

Fig. 2 Flow cytometric analysis of transfected S2 cells expressing the rabies virus glycoprotein. Flasks containing TC-100 medium were inoculated with transfected S2 cells to provide an initial concentration of 0.5×10^6 cells/ml. Samples were taken at 72 h after inoculation and cells were stained with anti-glycoprotein antibody conjugated to fluorescein. (**a**) Control (non-stained transfected cells); (**b**) transfected cells stained with antibody; (**c**) transfected cells grown in whole hemolymph (1% v/v) supplemented medium and stained with antibody

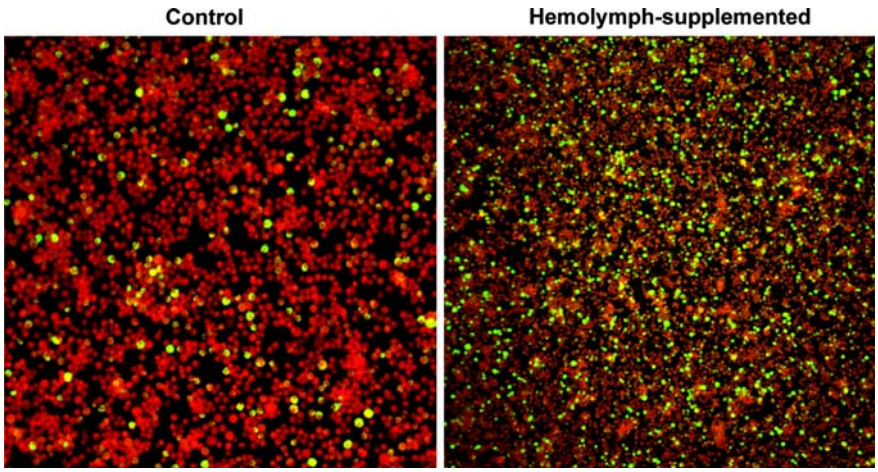

Fig. 3 Confocal images (magnification ×10) of transfected S2 cells expressing the rabies virus glycoprotein (GPV/PV), showing several degrees of expression intensity. Flasks containing TC-100 medium were inoculated with transfected S2 cells to provide an initial concentration of 0.5×10^6 cells/ml. Samples were taken at 72 h after inoculation and cells were stained with anti-glycoprotein antibody conjugated to fluorescein. (**a**) Control (transfected cells stained with antibody; (**b**) transfected cells grown in whole hemolymph (1% v/v) supplemented medium and stained with antibody

be observed, hemolymph addition increased cellular yields from 5.8×10^6 to 7.2×10^6 cells/ml, representing an increase of approximately 24%. No adverse effects of the hemolymph on cell growth seem to have taken place since both cultures displayed similar morphology throughout the experiments The effect of whole hemolymph (1% v/v) on the cell growth of transfected S2 cells expressing the rabies virus glycoprotein (GPV/PV) can be seen in Fig. 1b. The addition of whole hemolymph led to slightly lower cell growth in transfected S2 cultures (19% increase) in comparison with non-transfected S2 cell cultures (Fig. 1a). Consequently, a lower final cell yield (6.0×10^6 cells/ml) was achieved. TC-100 culture medium was employed in the experiments depicted in Fig. 1a and b. When SF-900 culture medium was used, no significant effect of the hemolymph on cell growth was observed (Fig. 1c).

Hemolymph effect on recombinant protein production (S2AcGPV)

In order to determine the hemolymph effect on recombinant protein production, cells were stained with fluorescent antibody and the cell number was determined by different techniques (FACS, confocal microscopy and ELISA). The flow cytometric analysis of transfected S2 cells (Fig. 2) shows that the number of fluorescent cells increased from 25.19% (Fig. 2a) to 42.54% (Fig. 2b) when the culture

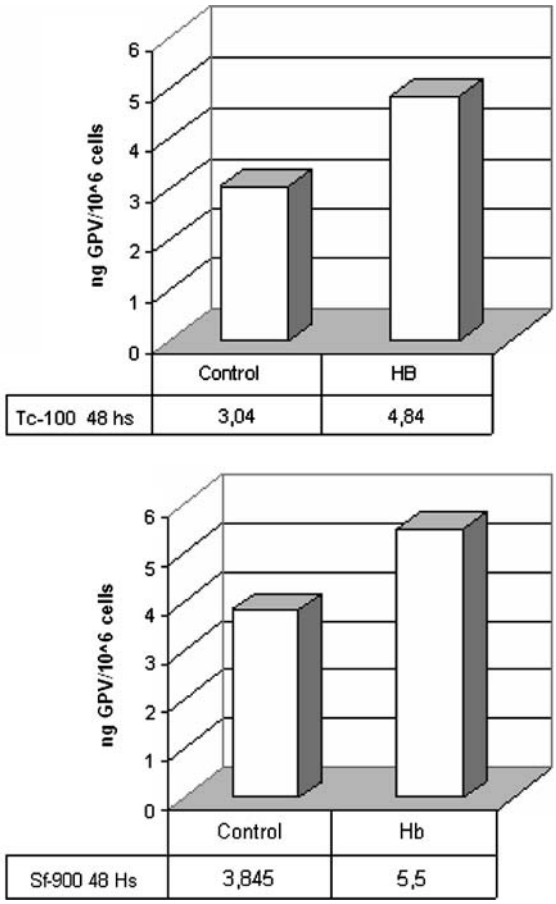

Fig. 4 Effect of culture medium on GPV production. S2 Cells were grown in TC-100 and SF-900 media. Control non-supplemented cultures and whole hemolymph supplemented cultures (1% v/v) were performed. Samples were taken daily. and the amount of glycoprotein in trimeric form was determined by ELISA. The results were expressed as ng GPV/10^6 cells

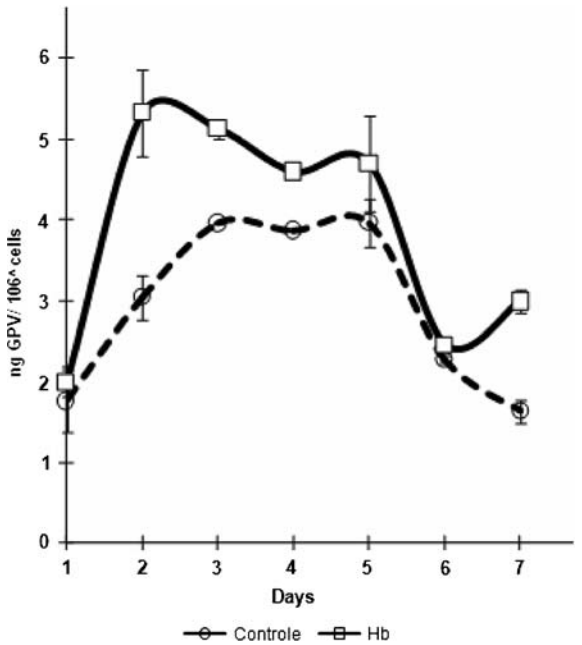

Fig. 5 Kinetics of GPV production in cultures of transfected S2 cells expressing the rabies virus G glycoprotein (GPV/PV). Flasks were inoculated with transfected S2 cells to provide an initial concentration of 1×10^6 cells/ml and whole hemolymph (1% v/v) was added to one of the cultures. Samples were taken daily, stained with anti-rabies G glycoprotein antibody and protein levels were quantified by ELISA. The results were expressed as ng GPV/10^6 cells

medium was supplemented with whole hemolymph (1% v/v), which corresponds to an increase of 68.8%.

Fluorescence of transfected S2 cells, cultivated in hemolymph supplemented and non-supplemented culture media, was also visualized by confocal microscopy (Fig. 3). Fluorescence intensity was greater in cells grown in hemolymph enriched cultures (58%) compared with cells grown in non-enriched cultures (20.14%).

Effect of culture medium on GPV production

S2 cell cultures (S2AcGPV) were conducted in Schott flasks (20 ml working volume) containing TC-100 and SF-900 media and the amount of proteins produced per cell was determined by ELISA. Even though cell number in SF-900 medium cultures was two to three times higher, the amount of GPV produced per cell was lower than in TC-100 medium cultures. Hemolymph addition to both cultures increased GPV production by an average of 42.8% (SF-900) to 59% (TC-100) (Fig. 4).

The kinetics of GPV production is depicted in Fig. 5. As illustrated, GPV production increased and achieved its maximum level during the exponential phase (at 48 h after inoculation) and decreased as the culture entered the stationary phase (Fig. 6), indicating that protein production may require cell mechanisms that are active during the cell growth phase.

Purification of hemolymph by gel filtration chromatography

Whole hemolymph was purified by gel filtration chromatography to determine the protein(s) involved

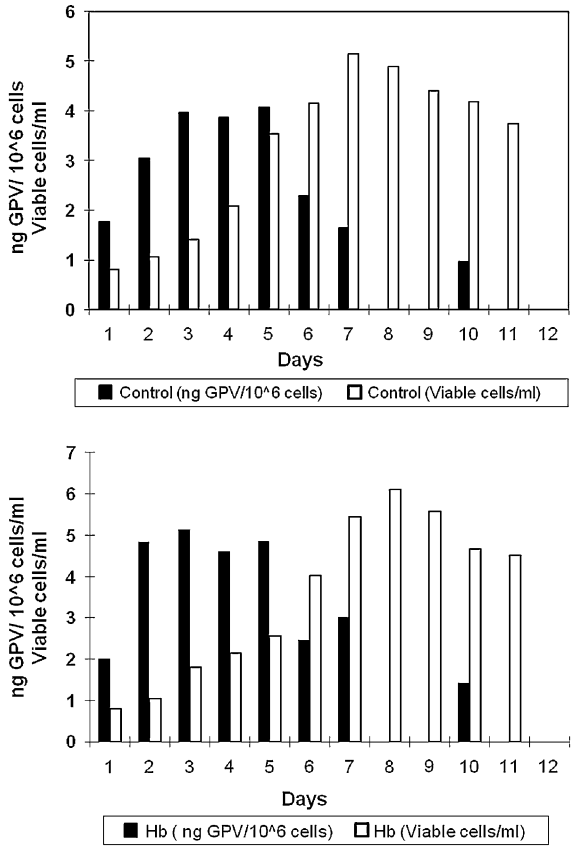

Fig. 6 Relationship between cellular growth and GPV production. Whole hemolymph (1% v/v) was added to one of the cultures. Samples were taken daily. Cells were stained with anti-rabies G glycoprotein antibody and protein levels were quantified by ELISA. The results were expressed as ng GPV/ 10^6 cells

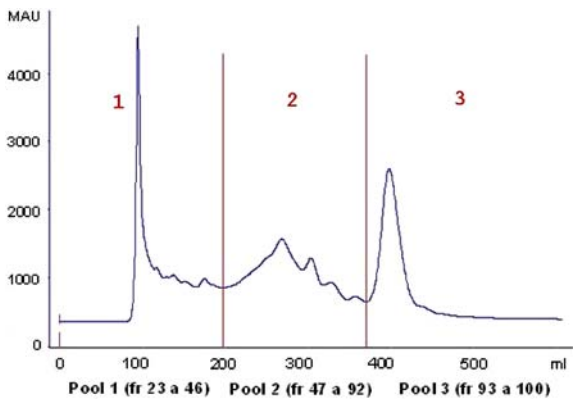

Fig. 7 Gel filtration chromatography of *Lonomia obliqua* hemolymph carried out in an AKTA Purifier chromatography system equipped with a Hi-prep 26/60 Sephacryl 200 column (Amersham Pharmacia Biotech) at a flow rate of 1 ml/min

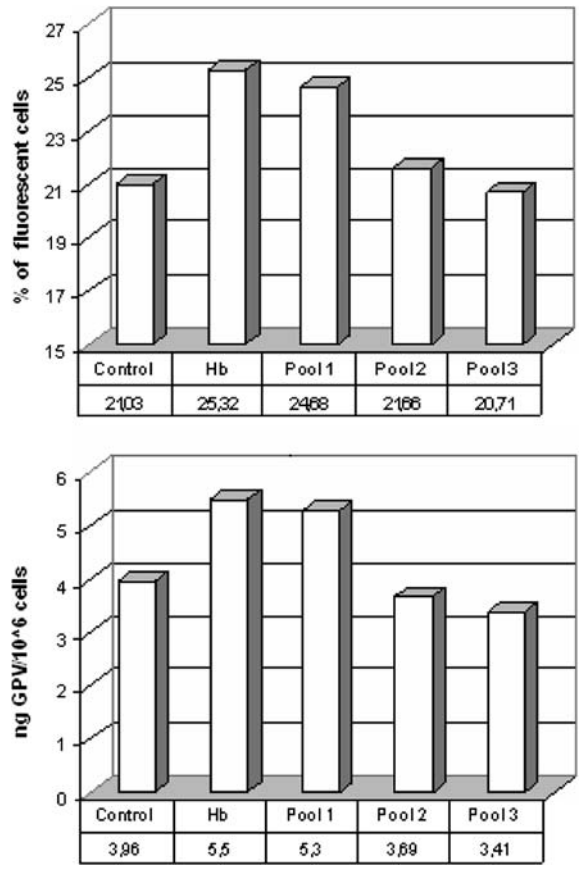

Fig. 8 Percentage of fluorescent S2 cells (**a**) and GPV production levels (**b**) after transfection with the gene of the rabies virus G glycoprotein (GPV/PV). The initial concentration of the cultures was 1×10^6 cells/ml. Cultures were supplemented with 1% (v/v) of whole hemolymph and 2% (v/v) of purified hemolymph fractions Control non-supplemented cultures were also performed Samples were taken after 72 h and cells were stained with anti-rabies G glycoprotein antibody The percentage of fluorescent cells was determined by flow cytometry (FACS) and the amount of GPV was quantified by ELISA

in the enhancement of recombinant protein production. Six milliliter of whole hemolymph were loaded onto a Sephacryl S200 gel filtration column and the collected fractions were then tested in transfected S2 cell cultures. Figure 7 depicts the chromatographic profile.

Effect of the hemolymph fractions on GPV production

Figure 8 shows GPV production (after 72 h) in cultures supplemented with whole hemolymph or its fractions, added at the time of inoculation. Samples

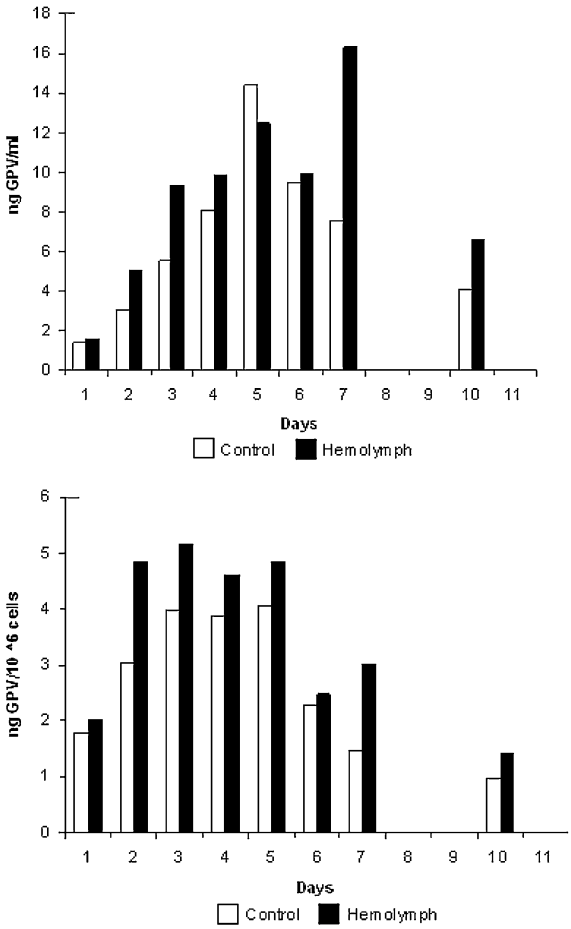

Fig. 9 Production of GPV/ml (**a**) and production of GPV/cell (**b**) by transfected S2 cells expressing the rabies virus G glycoprotein (GPV/PV). The cultures were supplemented with 2% (v/v) of purified hemolymph fractions (pool 1). Samples were taken daily and cells were stained with anti-rabies G glycoprotein antibody The amount of GPV produced was quantified by ELISA

were taken daily, beginning 72 h after inoculation. The percentage of fluorescents cells was determined by confocal microscopy and GPV production was determined by ELISA. As shown in Fig. 8, GPV production is directly proportional to the fluorescent cell number. While the addition of pools 2 and 3 resulted in poor performances in terms of GPV production, pool 1 supplemented culture yielded protein levels 34.8% higher than those observed in the control experiment. This a useful observation since the use of higher concentrations of the semi-purified protein may lead to even better GPV production performances.

The time courses of the amount of protein produced per cell and per ml are shown in Fig. 9. It can be observed that while the amount of GPV produced per cell remained practically unchanged between day 2 and day 5, the amount of GPV per ml increased in association with cell growth.

Discussion

In this article, we have identified and purified a protein from *Lonomia obliqua* hemolymph capable of stimulating the production of the rabies G glycoprotein, expressed in *Drosophila melanogaster* S2 cells. This is in agreement with the data published by Kanaya and Kobayashi (2000), showing that supplementation of the culture medium with *Bombyx mori* hemolymph led to an increase in recombinant luciferase production of up to 6,000 times in a polyhedrin-baculovirus system. According to Kanaya and Kobayashi (2000), the increase in recombinant protein production could be related to the increase in the baculovirus replication (10,000 times). Several proteins that stimulate viral replication have been described in the literature (Tanada et al. 1973; Hukuhara et al. 1987; Derksen and Granados 1988; Gallo et al. 1991; Xu and Hukuhara 1994. Nevertheless, the mechanism of action of the protein from *Lonomia obliqua* hemolymph may be different from the one of the protein from *Bombyx mori* hemolymph since the gene of GPV was transfected into S2 cells rather than expressed in a baculovirus system. The enhancing effect of some substances, such as sodium butyrate, on recombiant protein production has been attributed to direct action on DNA (Dorner et al. 1989; Palermo et al. 1991; Chang et al. 1999; Choi et al. 2005). However, the mechanism by which the protein in our study promotes GPV production remains to be solved. Previously, we isolated an anti-apoptotic protein from pool 1 of fractions of *Lonomia obliqua* hemolymph (Souza et al. 2005). We are currently investigating the possibility that this protein may be a multifunctional protein playing an essential role in the enhancement of GPV production observed. On the other hand, while the protein isolated in our study is a high molecular mass protein (about 51 kDa, Kanaya and Kobayashi characterized a 15 kDa protein).

Another relevant observation is that there is a relationship between cellular growth and the production of the protein in the cultures studied. As it can be observed in Fig. 8, the productivity per cell increased as cells grew and decreased as cells entered the stationary phase, indicating that the production of GPV may be directly related to cellular activity.

In conclusion, we have isolated a high molecular mass protein (pool 1 of chromatographic fractions) from *Lonomia obliqua* hemolymph, capable of increasing recombinant protein production by about 59%.

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